# Melamine, Cyanuric Acid, Ammeline, and Ammelide Analysis by Gas Chromatography / Mass Spectrometry

## cat.# 33254

**Melamine Analysis Kit (cat.# 33254) Includes:**

**GC Column**

| Description | cat.# |
|---|---|
| Rxi-5Sil MS w/5m Integra-Guard Column (30m, 0.25mm ID, 0.25µm) | 13623-124 |

**Standards:**

Volume is 1mL/ampul. Concentration is 1,000µg/mL in diethylamine:water (20:80).

| Description | cat.# |
|---|---|
| Melamine Stock Standard | 33247 |
| Cyanuric Acid Stock Standard | 33248 |
| Ammelide Stock Standard | 33249 |
| Ammeline Stock Standard | 33250 |
| Benzoguanamine Internal Standard | 33251 |
| Melamine and Related Analogs Stock Standard (1mL ampul containing 1,000µg/mL each: ammelide, ammeline, cyanuric acid, and melamine) | 33253 |

**Derivatization Reagent:**

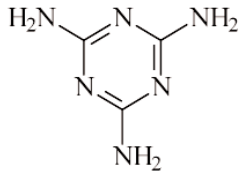
| Description | cat.# |
|---|---|
| 25g vial BSTFA w/ 1% TMCS | 35607 |

**Accessories:**

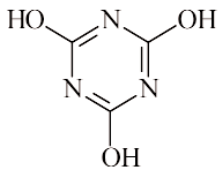
| Description | cat.# |
|---|---|
| 50mL empty centrifuge tube, 25-pk. | 26227* |
| 13mm, 0.45µm nylon syringe filter, 100-pk. | 26147* |

*Kit contains a 5-pack each of tubes and filters. 25-pks (cat.# 26227) and 100-pks (cat.# 26147) sold separately.

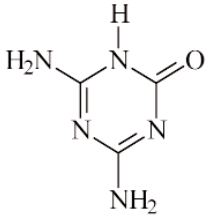
**Figure 1** Melamine and related compounds are rich in nitrogen and have been used to misrepresent protein levels in some food products.



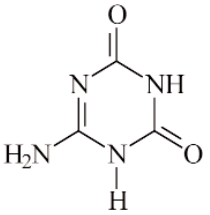
Melamine



Cyanuric Acid



Ammeline



Ammelide

### Introduction

The recent discovery of melamine in infant formula and pet food has raised industry, government, and public concerns about the potential for more widespread contamination. Melamine is not a legal food additive; it is a nitrogen-rich industrial compound used for plastics, flame-resistant products, and cleaning agents. However, melamine and related byproducts have been illegally added to food products in order to falsely represent the amount of protein present, since protein level in many products is determined using nonspecific assays for nitrogen content (Figure 1). While nontoxic alone at low doses, melamine does pose significant health risks, including kidney failure, when consumed in combination with cyanuric acid.

In response to the increasing need for more rigorous melamine and cyanuric acid testing, the US Food and Drug Administration (FDA) has set three different commodity-based minimum reporting levels (MRLs): 10µg/g for pet food, 2.5µg/g for human food, and 1µg/g for infant formula. The lower MRL level for infant formula was established because infants may rely on formula as their sole food source and since their renal systems are not yet fully developed.

### Method Overview

The products and step-by-step instructions included in this kit are designed to provide a complete solution for the GC/MS analysis of melamine and related compounds. The extraction and derivatization method described here was adapted from the FDA method, *GC-MS Screen for the Presence of Melamine, Ammeline, Ammelide, and Cyanuric Acid.*[1] Please refer to the FDA method for quality control (QC) requirements, reporting criteria, and semi-quantitative calculations.

Note that the FDA method guidelines differ based on the MRL for the commodity being analyzed; both high and low concentrations of standards and matrix spikes may be analyzed in the same analytical set for 10µg/g MRL commodities, but should be analyzed in separate sets for lower MRL commodities to reduce the potential for carryover contamination. For 2.5µg/g and 1µg/g MRL commodities, first analyze derivatized samples with the low matrix spike and standards. Then, if target analytes are detected above the MRL and semi-quantitative results are desired, new aliquots may be derivatized and analyzed with the high standards (and a high matrix spike, if desired). Instructions are provided here for the preparation of both high and low levels of QC matrix spikes and quantification standards for each MRL (Tables I and II). Example data are shown in Figures 2-7.

Finally, care must be taken to monitor for matrix interferences, which may complicate data collection and interpretation, as well as contaminate instrumentation. Analyzing derivatization reagent blanks at appropriate intervals is recommended. It may be necessary to perform a sample clean-up procedure to help reduce matrix interferences.

## Procedure

### Sample and Standard Preparation

Prepare stock standard solutions at the appropriate levels for the commodity being analyzed according to Table I. Next, use these stock solutions to make the actual samples and standards for analysis according to Table II. Some matrices have an analyte protecting effect, causing an increased response compared to analysis with solvent-only standards.[2,3,4] To avoid this effect, use matrix-matched standards, instead of solvent-based standards, for 2.5µg/g and 1µg/g MRL commodities, as shown in Table I. Matrix-matched standards are prepared in extracted analyte-free control matrix instead of solvent. Both matrix-matched and solvent-based standards must be derivatized according to the procedure in this method.

High and low matrix spikes are prepared using representative control sample matrix according to Table II and then extracted and derivatized. Low matrix spikes are prepared at the MRL to demonstrate adequate instrument sensitivity; thus, the low spike must be observed in order to report negative samples as below the MRL. High spikes are optional and can be useful indicators of major problems with sample extraction or system performance. A method solvent blank (20mL of 10/40/50 DEA/$H_2O$/ACN) should also be extracted to ensure there is no contamination from the reagents, nylon filter, or instrument system.

**Table I** Preparation of stock standard solutions.

| | 10µg/g MRL (Pet Food) | 2.5µg/g MRL (Human Food) | 1µg/g MRL (Infant Formula) |
|---|---|---|---|
| **Mixed Standard** Stock Standard 1,000µg/mL (cat.# 33253) | Target: 100µg/mL 1mL stock standard diluted to 10mL with 10/40/50 DEA/$H_2O$/ACN* | Target: 50µg/mL 500µL stock standard diluted to 10mL with 10/40/50 DEA/$H_2O$/ACN | Target: 10µg/mL 100µL stock standard diluted to 10mL with 10/40/50 DEA/$H_2O$/ACN |
| **Internal Standard** Benzoguanamine 1,000µg/mL | Target: 10µg/mL 100µL benzoguanamine diluted to 10mL with pyridine | Target: 1µg/mL 10µL benzoguanamine diluted to 10mL with pyridine | Target: 0.5µg/mL 5µL benzoguanamine diluted to 10mL with pyridine |
| **High Standard Prep Solution**** | Target: 10µg/mL 100µL Mixed Standard diluted to 1mL with 10/40/50 DEA/$H_2O$/ACN | Target: 1µg/mL 20µL Mixed Standard diluted to 1mL with control matrix extract | Target: 0.25µg/mL 25µL Mixed Standard diluted to 1mL with control matrix extract |
| **Low Standard Prep Solution**** | Target: 1µg/mL 10µL Mixed Standard diluted to 1mL with 10/40/50 DEA/$H_2O$/ACN | Target: 0.25µg/mL 5µL Mixed Standard diluted to 1mL with control matrix extract | Target: 0.1µg/mL 10µL Mixed Standard diluted to 1mL with control matrix extract |

*DEA/$H_2O$/ACN = diethylamine/deionized water/acetonitrile

**The use of matrix-matched standards, instead of solvent-only standards, is recommended for 2.5µg/g and 1µg/g MRL commodities. To prepare matrix-matched standards, use 1mL of filtered control matrix extract instead of 10/40/50 DEA/$H_2O$/ACN.

**Table II** Preparation of standards and matrix spikes for analysis (see text for extraction and derivatization procedures).

| | 10µg/g MRL (Pet Food) | 2.5µg/g MRL (Human Food) | 1µg/g MRL (Infant Formula) |
|---|---|---|---|
| **High Standard*** | Target: 1µg/mL Aliquot 50µL of High Standard Prep Solution and derivatize. | Target: 0.1µg/mL Aliquot 50µL of High Standard Prep Solution and derivatize. | Target: 0.25µg/mL Aliquot 50µL of High Standard Prep Solution and derivatize. |
| **Low Standard** | Target: 0.1µg/mL Aliquot 50µL of Low Standard and derivatize. | Target: 1µg/mL Standard Prep Solution and derivatize. | Target: 0.01µg/mL Prep Solution and derivatize. |
| **High Matrix Spike*** | Target: 50µg/g 250µL Mixed Standard in 0.5g matrix. Extract and derivatize. | Target: 5µg/g 50µL Mixed Standard in 0.5g matrix. Extract and derivatize. | Target: 2.5µg/g 125µL Mixed Standard in 0.5g matrix. Extract and derivatize. |
| **Low Matrix Spike** | Target: 10µg/g 50µL Mixed Standard in 0.5g matrix. Extract and derivatize. | Target: 2.5µg/g 25µL Mixed Standard in 0.5g matrix. Extract and derivatize. | Target: 1µg/g 50µL Mixed Standard in 0.5g matrix. Extract and derivatize. |

*The use of the high and low standards and high spikes differs by MRL. Refer to the Analytical Set section before preparing solutions.

## Matrix Extraction

Use the following procedure to extract test samples, matrix blanks, and matrix spikes. (Extracts from matrix blanks can also be used to prepare matrix-matched standard prep solutions according to Table I.)

- Weigh out 0.5g of each test or control sample into a 50mL centrifuge tube (cat.# 26227).
- Prepare high and low matrix spikes using control matrix according to the instructions in Table I *(omit this step for matrix control and test samples)*.
- Add 20mL of 10/40/50 DEA/$H_2O$/ACN solution.
- Mix well to wet entire sample.
- Sonicate for 30 minutes.
- Centrifuge for 10 minutes to pellet.
- Filter 2mL portion with 0.45µm nylon filter (cat.# 26147).

*Note: A 2mL portion is filtered to allow a second aliquot to be derivatized, if necessary.*

**Derivatization**

The derivatization procedure is necessary to allow melamine and related analogs to become volatile in the injection port of the gas chromatograph. Derivatize prepared samples and standards according to the following procedure.

- Transfer the following volumes to individual vials:
  - Sample supernatants: 200µL.
  - Matrix spike supernatants: 200µL.
  - Matrix-matched standards (high and low): 200µL.
  - Solvent-based standards (high and low): 50µL.
- Evaporate to dryness at 70°C with inert gas. *Verify that samples are dry; remaining water can disrupt the derivatization reaction.*
- Add 200µL pyridine.
- Add 200µL BSTFA with 1% TMCS derivatizing reagent.
- Add 100µL of benzoguanamine internal standard.
- Shake well or vortex for 10 minutes.
- Incubate at 70°C for 45 minutes.
- Inject into the GC/MS system.

**Analytical Set**

Standards should be injected both before and after the sample set for semi-quantitative analysis; however, the use of the high and low standards differs by the MRL. For 10µg/g MRL commodities, both low and high standards can be run in a single analytical set. For 2.5µg/g and 1µg/g MRL commodities, first analyze the samples with the low standards (do not inject the high standard). Then, if target analytes are detected above the MRL, new aliquots may be derivatized and analyzed separately with the high standards in a new analytical set. Low matrix spikes serve to establish detectability at the MRL and must be detected in order to report negative sample results as less than the MRL. The analysis of derivatization reagent blanks at appropriate intervals is recommended to detect carryover.

**GC/MS Parameters**

| | |
|---|---|
| Column: | Rxi®-5Sil MS, 30m x 0.25mm ID x 0.25µm with 5m Integra-Guard™ (cat.# 13623-124) |
| Instrument: | Shimadzu QP 2010 Plus |
| Injection mode: | splitless, 1 minute sampling time |
| Injection liner: | splitless, 3.5mm Gooseneck Splitless Line with wool (cat.# 2228-200.1) |
| Injection volume: | 1µL |
| Inlet temp.: | 280°C |
| Carrier gas: | helium |
| Linear velocity: | 37cm/sec., constant linear velocity |
| Oven temp.: | 75°C to 320°C @ 15°C/min. (hold 4 min.) |
| Detector: | MS |
| Transfer line temp.: | 290°C |
| Scan range: | 50-450 amu |
| Ionization: | EI, ion source at 190°C |
| Filament delay: | 8 minutes |
| Mode: | scan or SIM |

**Other liner choices by instrument:**

Agilent Gooseneck Splitless 4mm with wool (cat.# 22405)
PerkinElmer Auto SYS Splitless with wool (cat.# 20829)
Shimadzu Gooseneck Splitless with wool for 17A, 2010, 2014 (cat.# 22286-200.1)
Shimadzu Splitless with wool for 14A (cat.# 20863-200.1)
Thermo 4000/5000/6000 Splitless with wool (cat.# 20814-200.1)
Thermo TRACE, 8000, 8000 TOP, Focus SSL Splitless with wool (cat.# 20942-200.1)
Varian 1075/1077 4mm Splitless with wool (cat.# 20904-200.1)
Varian 1177 Gooseneck Splitless with wool (cat.# 21896-200.1)
Varian 1078/1079 Splitless with wool (cat.# 21711-200.1)

**MS Conditions** (SIM mode)

| Compound | Retention Time (min) | Target Ions | Reference Ions | Reference Ions | Reference Ions |
|---|---|---|---|---|---|
| Cyanuric Acid | 10.23 | 345 (100)* | 330 (36) | 346 (30) | 347 (15) |
| Ammelide | 11.07 | 344 (100) | 329 (58) | 345 (30) | 330 (16) |
| Ammeline | 11.76 | 328 (100) | 343 (79) | 329 (29) | 344 (24) |
| Melamine | 12.31 | 327 (100) | 342 (53) | 328 (30) | 343 (17) |
| Benzoguanamine | 14.54 | 316 (100) | 331 (68) | 332 (20) | 330 (9) |

*relative ion ratio

# Melamine Analysis Fortification and Extraction Sheet (QC Included)



| | Solvent Blank | Matrix Blank | Spike (_____µg/g) | Spike (_____µg/g) | Sample # | Sample # | Sample # | Sample # | Sample # |
|---|---|---|---|---|---|---|---|---|---|
| Actual weight (g) | | | | | | | | | |
| Fortification | | | | | | | | | |
| **Add** 20mL solvent (10:40:50 DEA:$H_2O$:ACN) | | | | | | | | | |
| **Sonicate** 30 min. start time:_____ end time:_____ | | | | | | | | | |
| **Centrifuge** 10 min. start time:_____ end time:_____ | | | | | | | | | |
| **Filter** supernatant (0.45µm nylon syringe filter) | | | | | | | | | |
| **Evaporate** filtered extract aliquot* to dryness (70°C & nitrogen gas) start time:_____ end time:_____ | | | | | | | | | |
| **Add** 200µL pyridine | | | | | | | | | |
| **Add** 200µL BSTFA with 1% TMCS | | | | | | | | | |
| **Add** 100µL benzoguanamine** | | | | | | | | | |
| **Shake** well (10 min.) | | | | | | | | | |
| **Incubate** at 70°C for 45 min. start time:_____ end time:_____ | | | | | | | | | |
| **Inject** (_____µL) | | | | | | | | | |
| Injection concentration (spikes) | | | | | | | | | |

* Aliquot volume varies by supernatant type.

| **Type** | **Aliquot** |
|---|---|
| Solvent-based standard | 50µL |
| Matrix-based standard | 200µL |
| Samples & spikes | 200µL |

** The recommended injection concentration of benzoguanamine (internal standard) is based on the MRL of the commodity being analyzed.

| **MRL** | **Benzoguanamine injection conc.** |
|---|---|
| 10µg/g (pet food) | 2µg/mL |
| 2.5µg/g (human food) | 0.2µg/mL |
| 1.0µg/g (infant formula) | 0.1µg/mL |

Method:

Analyst:

Date:

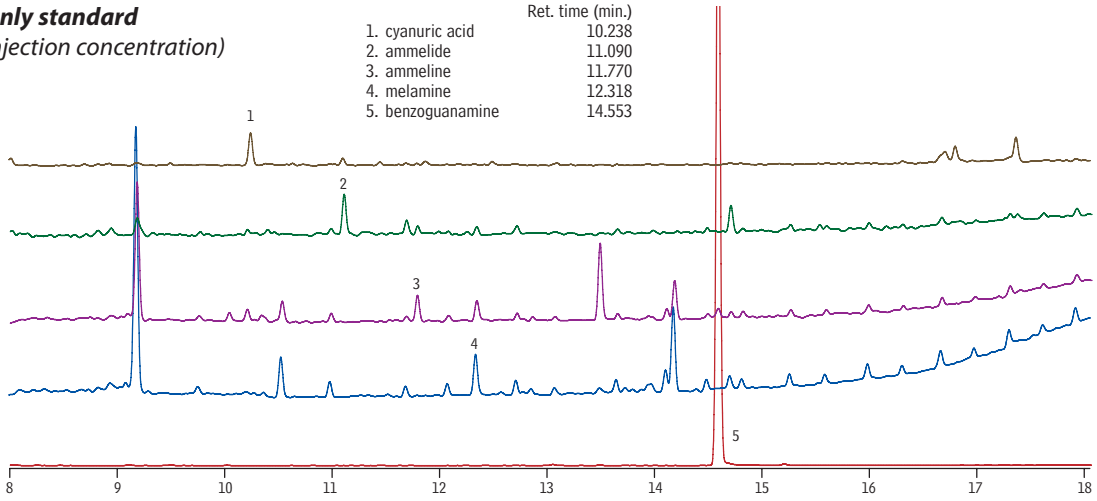
RESTEK

## Example Data

**Figure 2** Analysis of melamine and related compounds in infant formula (1µg/g MRL spike level).

***A. Solvent-only standard***
*(0.01µg/mL injection concentration)*

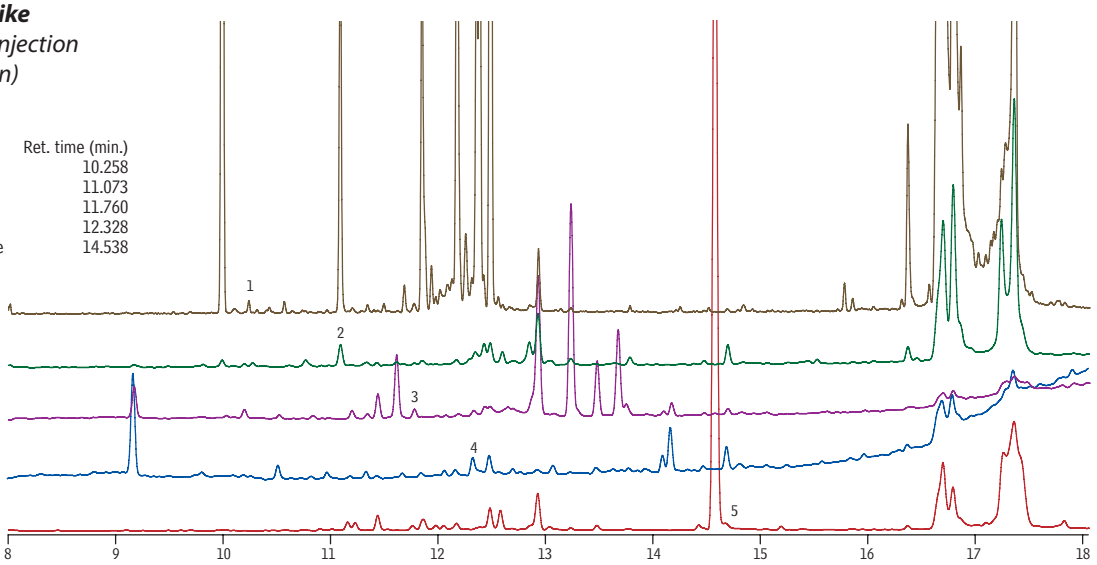
| | Ret. time (min.) |
|---|---|
| 1. cyanuric acid | 10.238 |
| 2. ammelide | 11.090 |
| 3. ammeline | 11.770 |
| 4. melamine | 12.318 |
| 5. benzoguanamine | 14.553 |




GC_FF01094 (A-E)

***B. Matrix spike***
*(0.01µg/mL injection concentration)*

| | Ret. time (min.) |
|---|---|
| 1. cyanuric acid | 10.258 |
| 2. ammelide | 11.073 |
| 3. ammeline | 11.760 |
| 4. melamine | 12.328 |
| 5. benzoguanamine | 14.538 |




GC_FF01095 (A-E)

| | |
|---|---|
| Column: | Rxi®-5Sil MS, 30m, 0.25mm ID, 0.25µm, w/ 5m Integra-Guard™ (cat.# 13623-124) |
| Instrument: | Shimadzu QP 2010 Plus |
| Sample: | A. Melamine and Related Analogs Stock Solution (cat.# 33253), Benzoguanamine (cat.# 33251) as tri-TMS derivatives, injection concentration: 0.01µg/mL<br>B. infant formula fortified at 1µg/g with Melamine, Related Analogs Stock Solution (cat.# 33253), Benzoguanamine (cat.# 33251), analyzed as tri-TMS derivatives, injection concentration: 0.01µg/mL |
| Inj.: | 1.0µL splitless (hold 1 min.), 3.5mm splitless inlet liner w/wool (cat.# 22286-200.1) |
| Inj. temp.: | 280°C |
| Carrier gas: | helium, constant linear velocity |
| Flow rate: | 1mL/min. |
| Oven temp.: | 75°C to 320°C @ 15°C/min. (hold 4 min.) |
| Det: | MS |
| Transfer line temp.: | 290°C |
| Ionization: | EI |
| Mode: | SIM (all method ions in table, only quantification ions were plotted) |

| Compound | Quant. ion | Qual. ion | Qual. ion | Qual. ion |
|---|---|---|---|---|
| cyanuric acid | 345 | 330 | 346 | 347 |
| ammelide | 344 | 329 | 345 | 330 |
| ammeline | 328 | 343 | 329 | 344 |
| melamine | 327 | 342 | 328 | 343 |
| benzoguanamine | 316 | 331 | 332 | 330 |

**Figure 3** Cyanuric acid spectrum

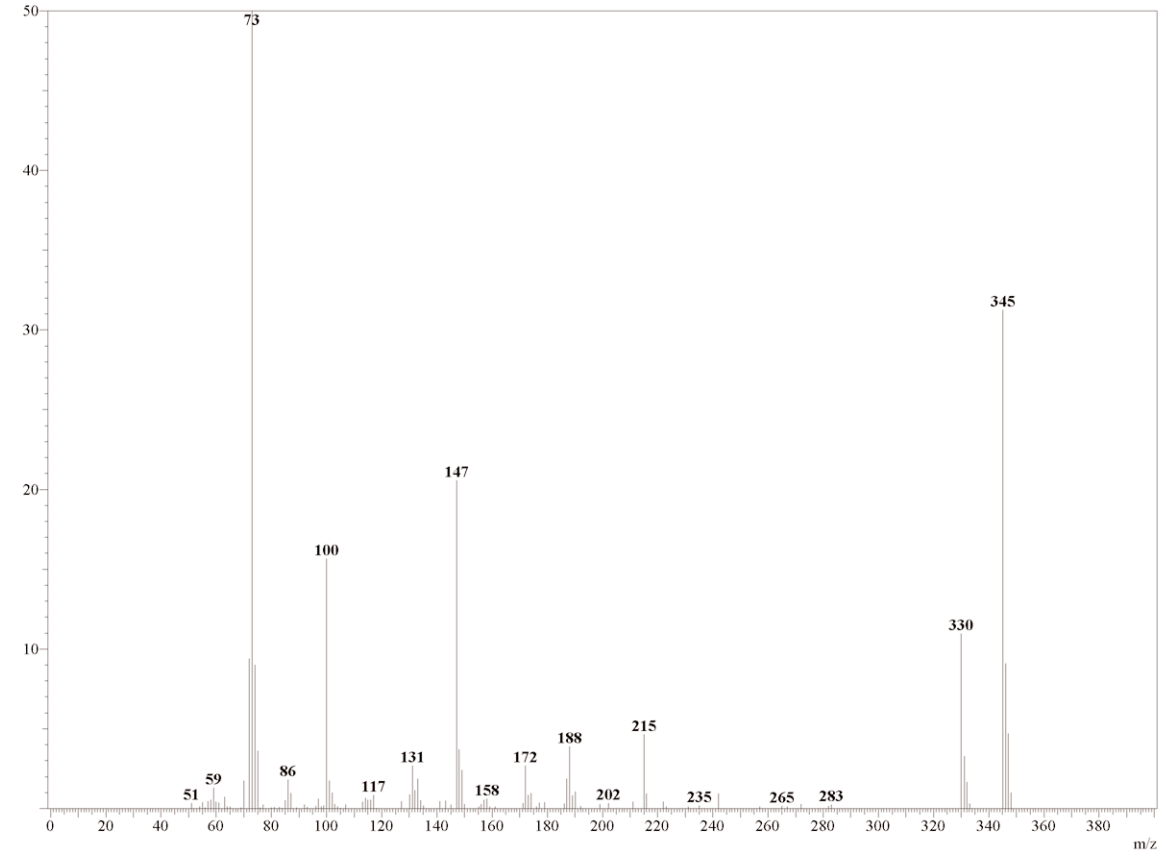



**Figure 4** Ammelide spectrum

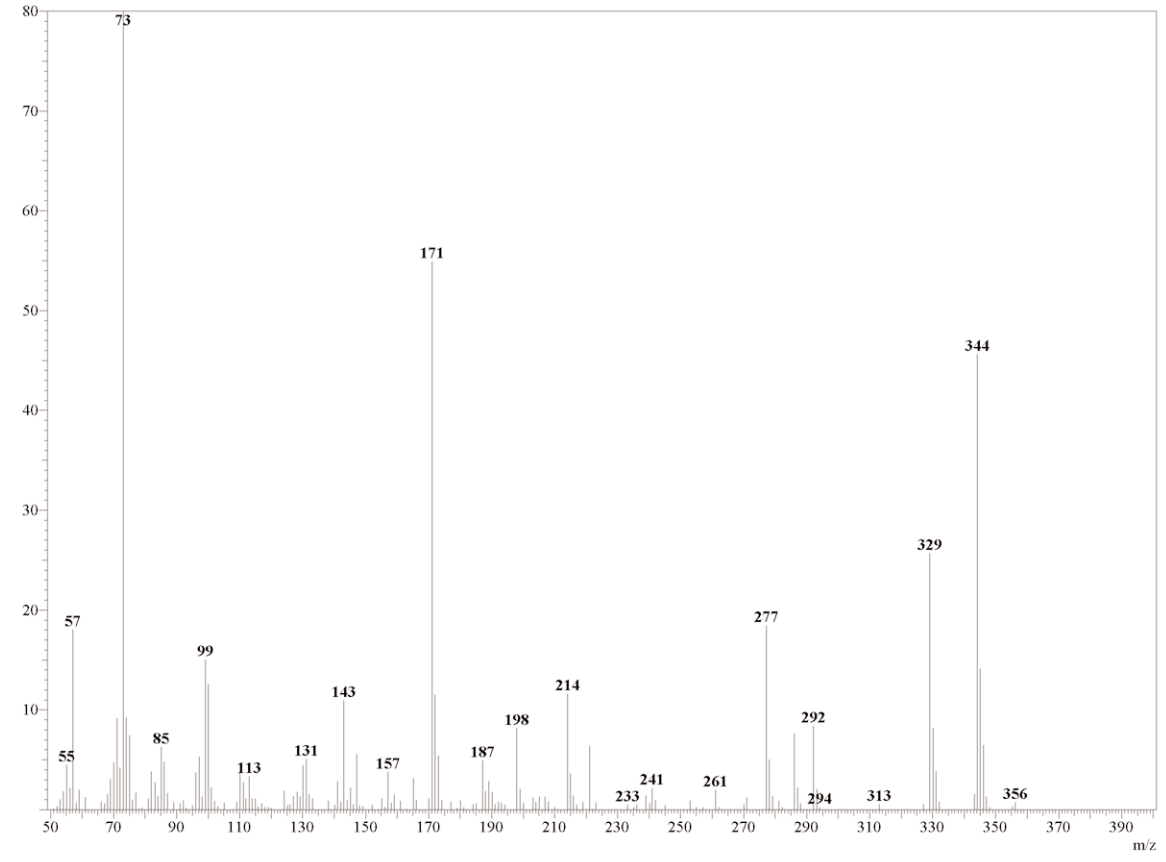


**Figure 5** Ammeline spectrum

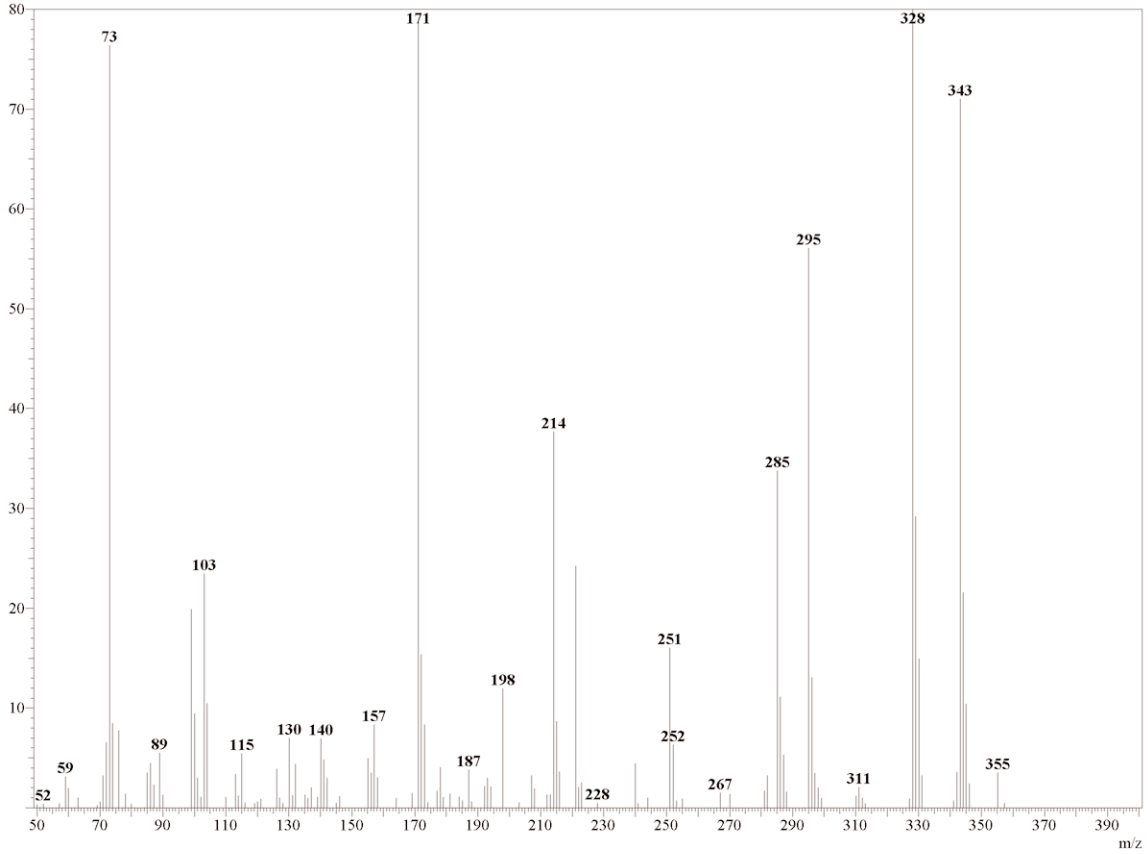



**Figure 6** Melamine spectrum

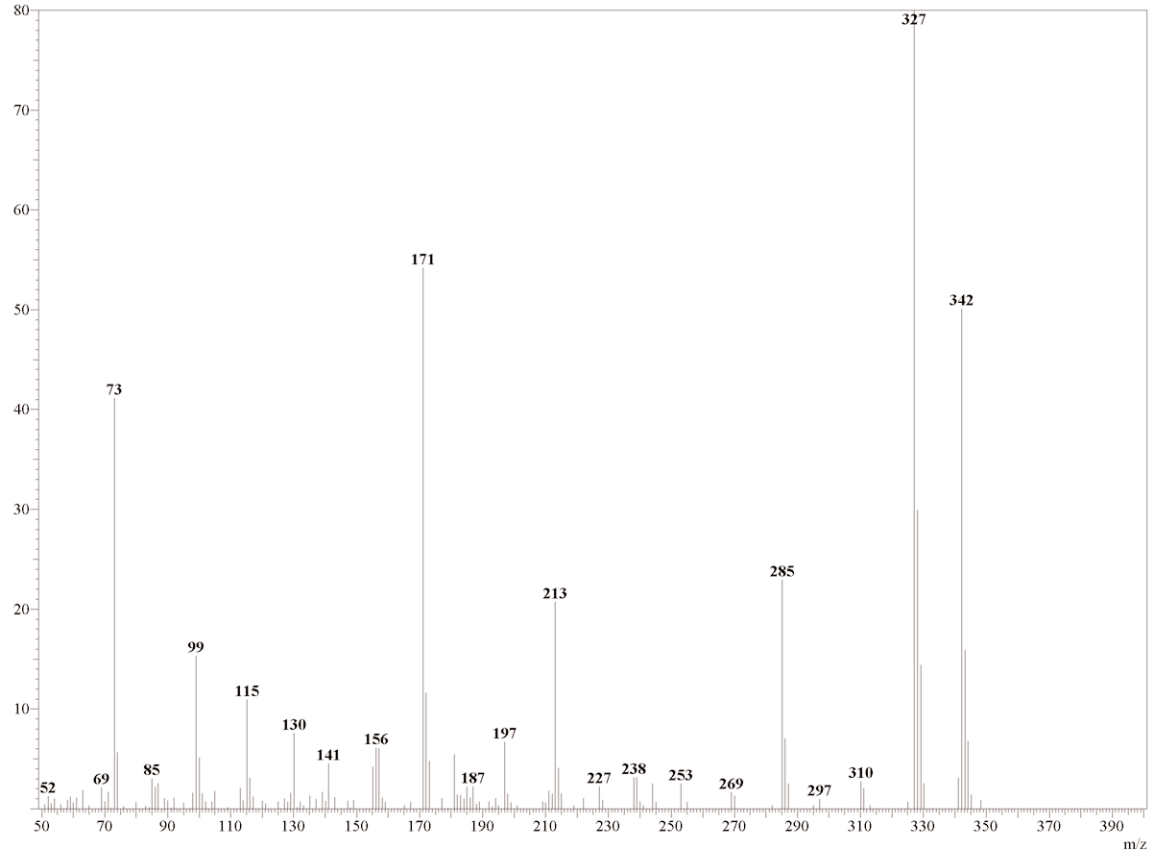
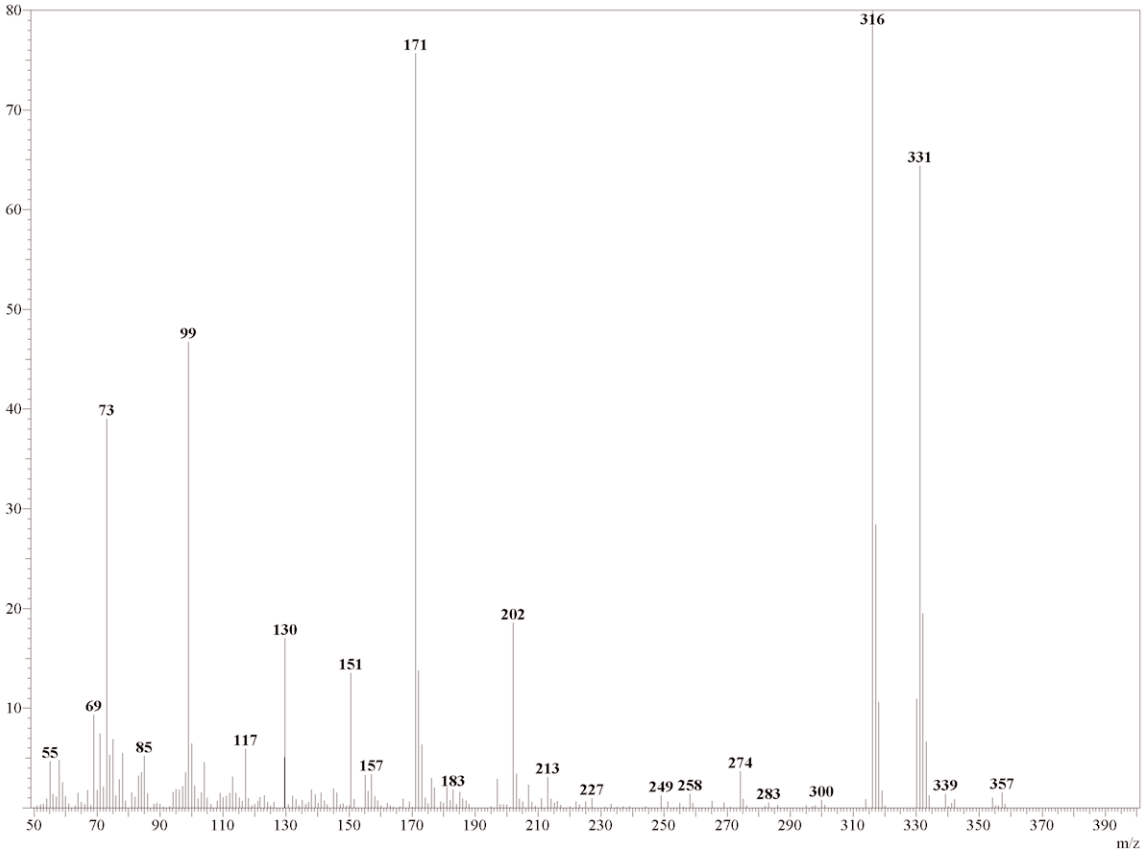


**Figure 7** Benzoguanamine spectrum





**References**

1 US Food and Drug Administration, October 2008, GC-MS Screen for the Presence of Melamine, Ammeline, Ammelide, and Cyanuric Acid, Laboratory Information Bulletin No. 4423, http://www.cfsan.fda.gov/~frf/lib4423.html.

2 C.F. Poole, J. Chromatogr. A 1158 (2007) 241.

3 T. Cajka, K. Maštovská, S.J. Lehotay, J. Hajšlová, J. Sep. Sci. 28 (2005) 1048.

4 K. Maštovská, S.J. Lehotay, M. Anastassiades, Anal. Chem. 77 (2005) 8129.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